DIFFERENCES IN AMINO ACID CONTENT BETWEEN CALFSKIN AND LIGAMENT ELASTINS*

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ABSTRACT

The amino acid composition of elastic tissue isolated from a calfskin was compared with the amino acid composition of bovine *ligamentum nuchae* elastin. The amino acid analysis of hide elastin depended upon the method of preparation indicating that at least two definite fractions were present. Neither fraction resembled the ligament elastin in amino acid content.

INTRODUCTION

Elastic tissue components having remarkably similar histological and physical properties have been found in the connective tissue of many organs. All the elastins whose amino acid contents have been determined seem quite similar in chemical composition although some minor differences have been shown. The present knowledge of these elastins and their relationship to the other connective tissue proteins was adequately summarized by Partridge (1).

The elastic fibers from calfskins and cattlehides have the characteristic behavior of the other elastins described by Partridge. However, when Bowes and Kenten (2) compared paper chromatograms of hydrolyzates of elastins prepared from *ligamentum nuchae* and the skin of an ox, they found differences in the amino acid composition. They could not determine whether these differences were due to differences in the protein or to the methods by which the protein was isolated. In the meantime, Smith et al. (3) reported analyses for the amino acid composition of elastin from human skin which were remarkably similar to the amino acid composition of elastin from bovine *ligamentum nuchae*. In the isolation of the elastic tissue from hides, Mellon and Korn (4) were able to retain it intact as a three-dimensional network while thoroughly removing all non-

elastic materials. The present study reports the amino acid composition of elastic tissues prepared from calfskins by several treatments. These have been compared with the amino acid content of ligament elastin to show the complex nature of the elastic tissue of hides.

EXPERIMENTAL

Preparation of Ligament Elastin.—The *ligamentum nuchae* was obtained from the neck of a freshly killed steer. The tissue was extracted in five percent sodium chloride solution for three days, washed in water, then shredded. The shredded ligament was divided into two portions, and each portion was purified by a different procedure.

Portion number one was extracted with five percent salt solution for an additional two days. The salt was washed out with distilled water, and ligament pieces were autoclaved for three hours. The supernatant liquor was discarded and the elastin washed thoroughly to remove dispersed materials. The elastin was then autoclaved in fresh distilled water for another two and one-half hours. After washing thoroughly, the sample was dried and defatted by extracting several times with boiling acetone and once with ether. After equilibration with the air for a week, the sample was ground to a fine powder. This is reported in Table I as Sample A.

Portion number two of the ligament was extracted with one-half saturated lime water for two days, drained thoroughly and extracted again for one day with fresh one-half saturated lime water. After washing thoroughly to remove the lime, the ligament was boiled in distilled water for one hour. The boiling in fresh distilled water was repeated another three times. The sample was then boiled in ten percent acetic acid for two hours, extracted with five percent hydrochloric acid for two hours, washed thoroughly, and dehydrated by boiling first in alcohol and then ether. After equilibration with the air for a week, the sample was ground to a fine powder. This is reported as Sample B.

Preparation of Skin Elastins.—The grain elastin samples were prepared from calfskins which had been unhaired and pickled in a commercial tannery. One portion of pickled calfskin was extracted with six percent sodium acetate solution at pH 6.5, washed with water and autoclaved for two hours in distilled water. After washing out the dispersed materials, the residual tissue was again autoclaved in distilled water for one hour. After thorough washing the dense elastic tissue from the grain area was separated from the more filamentous elastic tissue of the corium area by the technique of Hoover et al. (5). This elastic tissue was dehydrated with acetone and ground to a fine powder. This is reported as sample C.

A second portion of pickled calfskin was extracted with ten percent sodium chloride solution to remove the pickling acid. It was adjusted to pH 8.0 with

sodium hydroxide and autoclaved at 120°C. for two hours. After removal of the dispersed materials, the elastin residue was again autoclaved in a 0.01 M. phosphate buffer at pH 8.0 for an additional two hours. The elastin was separated, dried and ground like the previous sample. This is reported as Sample D.

A portion of the Sample D elastin preparation was treated further by extraction with one percent potassium hydroxide solution, water, ten percent acetic acid, and five percent hydrochloric acid, in that order. The elastin residue was then washed free of chloride ion and dried like the previous samples. It is reported as Sample E.

The potassium hydroxide, acetic acid, and hydrochloric acid extracts were combined and neutralized with sodium hydroxide. A precipitate formed which was recovered by centrifugation, resuspended in water and dialyzed with running water at room temperature. The suspension was then centrifuged, the liquid was decanted, and the solids were lyophilized. This is reported as Sample F.

Analysis.—The moisture content of each sample was determined at the time of analysis, and all results are given on a moisture-free basis. Nitrogens were determined by the Kjeldahl method.

The elastins were prepared for amino acid analysis by hydrolyzing 0.2 gm. samples in 6 *N* hydrochloric acid for 18 hours. The acid was evaporated off, and the samples were dissolved in 0.1 *N* hydrochloric acid. Aliquots of these solutions were diluted with column buffer to the proper concentration for analysis. The analysis was run on a Piez-Morris (6) ion exchange column employing a continuous gradient elution buffer. All 19 amino acids could be determined in a single run, and all the runs were made on the same column. Each value reported is an average of the values from at least two runs.

RESULTS AND DISCUSSION

Analysis of elastins from various sources has produced an abundance of data which appear to be quite similar yet show many small differences. Tables of these comparisons appear in the literature (1), but only the recent values by Partridge (1) for ligament elastin will be used here to illustrate the high degree of agreement between our values and his. The first three columns in Table I contain the data for the grams of amino acid per 100 grams of the moisture-free elastin from *ligamentum nuchae.* The first column is the data of Partridge. The second and third columns are the data from Samples A and B of our preparations of elastin from ligament. These three columns of data demonstrate the reproducibility of ligament elastin composition for samples produced from different animals and by different methods.

The values presented by Smith (3) on the elastin from human skin are in good agreement with these data for elastin from *ligamentum nuchae.* Most of the differences can be explained by the corrections for decomposition and incomplete hydrolysis applied by Smith.

TABLE I

AMINO ACID COMPOSITION OF ELASTINS

(g./100 g. protein)

Amino Acid	Ligament			Calfskin Preparations			
						Extracted C	
	Literature Partridge (1)	Experimental		pH 6.5 C	pH 8.0 D	Insoluble E	Soluble F
		A	B				
aspartic	1.1	1.0	0.9	3.2	4.7	3.0	7.2
threonine	1.1	1.2	1.1	2.1	2.4	1.8	3.4
serine	0.9	1.1	0.9	2.2	2.6	1.9	3.6
glutamic	2.4	2.6	2.4	5.2	7.3	4.9	9.6
proline	13.5	15.4	15.4	8.8	7.6	8.4	3.6
glycine	26.7	27.9	28.4	16.2	12.9	15.8	4.3
alanine	21.3	22.1	22.4	13.3	11.0	13.2	4.9
cystine		0.3	tr.*	0.2	0.4	0.2	0.4
valine	17.7	17.2	18.2	11.4	9.2	10.6	5.1
methionine		tr.*	0.0	0.6	1.2	0.8	1.4
isoleucine	3.8	3.7	3.8	3.9	3.9	3.4	4.3
leucine	9.0	9.1	9.3	8.6	8.6	8.1	8.8
tyrosine	1.5	1.8	1.8	2.5	2.9	2.3	3.6
phenylalanine	6.2	5.9	5.7	5.1	4.9	4.9	4.6
lysine	0.5	0.6	0.5	3.0	3.3	2.3	4.7
histidine	0.1	tr.*	tr.*	0.8	1.2	0.8	1.6
arginine	1.3	1.3	1.1	3.8	4.1	2.8	5.8
hydroxyproline	1.6	1.2	1.5	1.5	1.4	1.2	0.1
hydroxylysine		tr.*	tr.*	tr.*	tr.*	tr.*	tr.*
total	108.7	112.4	113.4	92.4	89.6	86.4	77.0
% nitrogen	16.8	17.2	17.1	15.8	13.8	15.0	12.6

*tr = trace

The data for the elastic tissue obtained from calfskins show a greatly different amino acid composition from that of ligament elastin as well as a variation of this composition depending upon the method of preparation. Since samples C, D, and E consist only of the residual three-dimensional fibrous network from the grain area, our results might differ from those determined by analysis of total insoluble materials obtained by centrifuging autoclaved tissues. Most workers use the total amount of insoluble material which could be obtained by centrifuging the autoclaved materials. This may not make an appreciable difference when the tissue is the *ligamentum nuchae,* but it could produce an appreciable difference in skin and other tissues. In our method of preparation the fine insoluble materials are washed away, and only the coarse fibrous network remains.

The data for Sample C, which we believe to be our purest preparation of elastin from calfskin, when compared with those for the ligament elastin, show: much lower contents of proline, glycine, alanine, and valine; about the same con-

centrations of isoleucine, leucine, and phenylalanine; and higher concentrations of most of the other amino acids. These results confirm the findings of Bowes and Kenten (2) whose paper chromatographic analysis of ox hide elastin indicated that appreciably more of the dicarboxylic and basic amino acids were present in the hide elastin than in ligament elastin.

When calfskin samples were autoclaved at the more alkaline pH of 8.0, the elastin residue appeared to look whiter than when the samples were autoclaved at the lower pH of 6.5. The analysis of Sample D prepared by autoclaving at pH 8.0 is shown in column 5. The values are quite different from the other calfskin elastin (Sample C) and also from the ligament elastin. Since many of its amino acids were present in higher concentrations than in the other calfskin elastin sample, it appeared that it might contain additional proteins.

This alkaline-prepared elastin was extracted at room temperature in an attempt to separate its components. An additional extraction with one percent potassium hydroxide solution had little effect, so extractions with acids were made. An extraction with ten percent acetic acid removed a component which could be precipitated by neutralization. A subsequent extraction with five percent hydrochloric acid removed very little additional material; about one-quarter of the material was removed by the acid treatment. This material, after dialysis to remove salts and lyophylization to dry it, had the amino acid composition reported as Sample F. The content of acidic and basic amino acids is much higher, and the content of proline, glycine, alanine, and valine much lower than for the elastin residue C made at pH 6.5.

The elastin residue from this extraction has the amino acid composition shown for Sample E. This is very closely related to the composition of the other calfskin elastin in Sample C. This indicates that the elastic tissue obtained by autoclaving at pH 8.0 is not a simple protein but is composed of at least two proteins, one of which is identical in amino acid content with the elastin prepared by autoclaving at pH 6.5.

The data show that the nitrogen contents are appreciably lower for the calfskin elastin samples and are lowest for the solubilized fraction from the alkaline-prepared elastin. This would indicate that an appreciable amount of nonamino acid material might be associated with this elastic tissue and would account for the lower percentage of total amino acids recovered from the hydrolyzates. For all the calfskin elastic tissues a noticeable amount of insoluble fibrous material persisted in the hydrolyzing medium. This was seen as soon as the sample was dispersed in the hydrolyzing acid and before appreciable color formed, and therefore it is not believed to be due to humin formation. Analysis of a small amount of this material showed that it contained around two percent nitrogen. It was not present in the ligament elastin. An earlier study (5) of the elastic membranes from calfskins showed the presence of carbohydrates by the anthrone method.

CONCLUSIONS

The elastic tissue obtained from the papillary region of calfskins by autoclaving at pH 8.0 is a complex protein. It can be separated by acetic acid extraction into two fractions, both of which are considerably different in amino acid composition from the elastin obtained from *ligamentum nuchae* or human skin. One of these fractions is identical with the elastic tissue produced from calfskins by autoclaving at pH 6.5.

It appears that some specific action or sequence of treatment is required to liberate the soluble fraction, because if it were originally acid extractable it would have been washed out when the pickling acid was removed. It is solubilized by autoclaving at pH 6.5 but not by autoclaving in pH 8.0 buffers, although this treatment renders it acid soluble. The buffer salts may have a greater effect on the tissue than was expected.

These observations strengthen the belief that calfskin elastic tissue is different from and much more complex than the elastic tissue from ligaments and human skin.

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DISCUSSION

The discussion leader on this paper will be Dr. Ross Donovan of Canada Packers Limited.

DR. DONOVAN: I'd like to thank Dr. Mellon for this paper and start the discussion by asking some questions. Dr. Mellon, Partridge, Piez and others have shown that elastins change in composition with the age of the animal from which they are taken. Would you say that the differences you have observed may be due to differences in the ages of the specimens you took, or are they strictly tissue differences?

DR. MELLON: We expect that these are tissue differences. For all of the elastins which Partridge has reported on, and these involve tissues of all ages,

the variations in the content of the various amino acids are very slight compared with the differences in the four amino acids: glycine, alanine, valine and proline, which we have shown here today.

DR. DONOVAN: Thank you. Questions from the floor?

MR. SHIVAS (Robson-Lang Leathers Company): What percentage of elastin is there in calfskin?

DR. MELLON: On the total weight of calfskin, roughly 0.2–0.3 percent. The grain area would run higher than this because the elastin is concentrated in the grain area. In the center of the grain it approaches 2 percent. Of the total elastin material in the calfskin, roughly 90 percent is in the grain, the other 10 percent is distributed generally through the corium.

DR. SHAW (Rohm and Haas): How would you expect this elastin to affect leather manufacture? Does it affect the properties of the leather?

DR. MELLON: We thought the elastin network possibly would affect the properties, and we set up a project at the University of Cincinnati to remove the elastin by enzymatic treatment. The results indicated that the elastin removal had a slight effect on the stretchability and on some of the physical properties. There was no difference great enough to make any commercial difference. Elastin when dry is quite brittle and we feel that the staking procedure breaks the network up sufficiently so that it does not affect the properties at all.

MR. H. DOBERTHEIN (Robson-Lang Leathers): What distinguishes elastin from collagen?

DR. MELLON: The main physical characteristic is its elasticity when it is wet. It is very elastic — just like a rubber band. There is also a difference in the amino acid content — the elastins have only one percent, or one and one-half percent of hydroxyproline, whereas collagen has somewhere around 13 percent.

DR. DONOVAN: Thank you. If there are no more questions, I'd like to thank Dr. Mellon and his group for showing us once again that there's more to skin than meets the eye.